Snf1-Dependent and Snf1-Independent Pathways of Constitutive *ADH2* Expression in *Saccharomyces cerevisiae*

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ABSTRACT

The transcription factor Adr1 directly activates the expression of genes encoding enzymes in numerous pathways that are upregulated after the exhaustion of glucose in the yeast *Saccharomyces cerevisiae*. *ADH2*, encoding the alcohol dehydrogenase isozyme required for ethanol oxidation, is a highly glucose-repressed, Adr1-dependent gene. Using a genetic screen we isolated >100 mutants in 12 complementation groups that exhibit *ADR1*-dependent constitutive *ADH2* expression on glucose. Temperature-sensitive alleles are present among the new constitutive mutants, indicating that essential genes play a role in *ADH2* repression. Among the genes we cloned is *MOT1*, encoding a repressor that inhibits TBP binding to the promoter, thus linking glucose repression with TBP access to chromatin. Two genes encoding proteins involved in vacuolar function, *FAB1* and *VPS35*, and *CDC10*, encoding a nonessential septin, were also uncovered in the search, suggesting that vacuolar function and the cytoskeleton have previously unknown roles in regulating gene expression. Constitutive activation of *ADH2* expression by Adr1 is *SNF1*-dependent in a strain with a defective *MOT1* gene, whereas deletion of *SNF1* did not affect constitutive *ADH2* expression in the mutants affecting vacuolar or septin function. Thus, the mutant search revealed previously unknown Snf1-dependent and -independent pathways of *ADH2* expression.

T HE yeast *Saccharomyces cerevisiae* has a sophisticated system for transducing information regarding nutrient availability to the nucleus (Gancedo 1998; Carlson 1999). Glucose, the preferred source of energy, is oxidized to produce carbon dioxide and ethanol during fermentative growth. During this phase of growth the transcription of genes involved in many nonfermentative pathways is repressed, as are genes required for the utilization of less-preferred fermentable substrates, such as galactose, sucrose, and maltose. When glucose is exhausted, these genes are activated to allow the cell to use alternative carbon sources for growth and energy production (Schuller 2003), an adaptive change in metabolism that occurs during the diauxic transition. This change in metabolism is accompanied by a massive reprogramming of gene expression (DeRisi *et al.* 1997).

The diauxic transition is regulated by the Snf1 protein kinase, the yeast homolog of the mammalian AMP-activated protein kinase (AMPK) (Hardie *et al.* 1998). Snf1 is part of a kinase complex whose activity is stimulated by low glucose concentration. The activity of the Snf1 kinase complex is regulated by Glc7.Reg1.Bmh, a type I protein phosphatase complex (Sanz *et al.* 2000; Dombek *et al.* 2004); three targeting subunits (Schmidt and McCartney 2000); and three upstream kinases (Hong *et al.* 2003). Many of the genes whose expression is Snf1-dependent encode regulatory proteins, such as protein kinases, protein phosphatases, and transcription factors, suggesting that Snf1 acts through a complex regulatory cascade (Young *et al.* 2003).

Adr1 and Cat8 are two transcription factors that act downstream of Snf1 to activate nonfermentative metabolic pathways (Schuller 2003). Adr1 and Cat8 act both independently and synergistically to regulate >100 genes after the diauxic transition (Young *et al.* 2003; Tachibana *et al.* 2005). One of the Snf1-dependent genes activated by Adr1 and Cat8 is *ADH2,* encoding alcohol dehydrogenase II, the isozyme that catalyzes the first step in ethanol oxidation. No DNA-binding repressors of *ADH2* transcription have been identified (Irani *et al.* 1987). Instead, *ADH2* expression is repressed by the absence of active Snf1, which is kept in an inactive state in the presence of glucose by an active Glc7.Reg1.Bmh complex (Dombek *et al.* 1993, 2004). Activation (derepression) of *ADH2* expression requires the cooperative binding of Adr1 and Cat8, leading to synergistic activation when both factors are present (Walther and Schuller 2001; Tachibana *et al.* 2005). Snf1 regulates both the expression and the activity of Cat8 (Rahner *et al.* 1999; Charbon *et al.* 2004).

Snf1 may regulate Adr1 activity at more than one level as well. Adr1 binding to chromatin is regulated by Snf1 (Young *et al.* 2002), perhaps through modification of

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chromatin since Adr1 can bind constitutively to UAS1 in the *ADH2* promoter if two histone deacetylases, Rpd3 and Hda1, are absent (VERDONE *et al.* 2002). However, full transcriptional activation of *ADH2* does not occur even when Adr1 is bound to the promoter because TBP is not recruited, suggesting a second glucose-regulated step in *ADH2* transcription.

The original genetic selection that was used to identify genes that cause constitutive *ADH2* expression yielded semi-dominant *ADR1^c* alleles (mutations in a cAPK phosphorylation motif; CHERRY *et al.* 1989) and *ADH2* promoter mutations (CIRIACY 1976, 1979; WILLIAMSON *et al.* 1981; RUSSELL *et al.* 1983). Both classes of mutation act independently of glucose repression. Subsequently, *ADR1*-independent (*CRE1/SPT10/SUD1*, *CRE2/SPT6/SSN20*; DENIS 1984), partially *ADR1*-independent (*ADR7*, *ADR8*, and *ADR9*, KARNITZ *et al.* 1992), and strictly *ADR1*-dependent recessive constitutive *ADH2* mutants (*REG1*) were identified (DOMBEK *et al.* 1993, 1999).

Mutations in *REG1* and *GLC7* allow glucose-insensitive *ADH2* expression only in the presence of *ADR1* and *SNF1*, suggesting that constitutive *ADH2* expression in the absence of an active PP1 complex requires the same components that are used normally during derepression (DOMBEK *et al.* 1993, 1999). Since loss of either *REG1* or *GLC7* causes only partial release from repression it is likely that other genes are involved in regulation of *ADH2* expression in the presence of glucose. However, since both Adr1 and Cat8 are regulated at both the transcriptional and the post-translational levels (DENIS and GALLO 1986; BLUMBERG *et al.* 1987; RAHNER *et al.* 1996; SLOAN *et al.* 1999), mutations in a single gene in the repression pathway might not cause constitutive *ADH2* expression. This interpretation is supported by two observations. First, mutations in *REG1* cause an elevation in both *ADR1* expression and activity (DOMBEK *et al.* 1993). Second, mutations in *GLC7* cause constitutive *ADH2* expression only when *ADR1* is modestly overexpressed (DOMBEK *et al.* 1999). These observations suggest that additional levels of control over repression act directly upon Adr1 or its expression. Although the level of Adr1 protein increases during derepression, elevated Adr1 levels alone are insufficient for full *ADH2* activation (DOMBEK and YOUNG 1997; SLOAN *et al.* 1999).

To identify additional genes required for repression of *ADH2* expression, we used a strain containing four copies of *ADR1* and an *ADH2/lacZ* reporter gene. In this strain glucose repression of *ADH2* expression is maintained even though Adr1 protein levels in repressed cells are the same as in derepressed cells (SLOAN *et al.* 1999). We assumed that overproduction of Adr1 might overcome the influence of transcriptional repression of *ADR1* expression on *ADH2* activation and allow us to identify new genes involved in the regulation of Adr1 activity. By screening for constitutive *ADH2/*β-galactosidase activity we isolated over 100 mutants representing at least 12 complementation groups. Mutants in several of these complementation groups had additional phenotypes, including temperature sensitivity, invertase constitutivity, and abnormal cell morphology. We cloned *MOT1*, *FAB1*, *VPS35*, and *CDC10* by genetic complementation of the mutant defects. These genes have known functions in other pathways but had not previously been shown to be involved in glucose repression of gene expression.

MATERIALS AND METHODS

Yeast strains and plasmids: The *S. cerevisiae* strains used in this study are listed in Table 1. Multicopy *ADR1* strain VBY20 was obtained by integrating an *ADH2/GFP* reporter into the *URA3* locus of JSY24 and crossing a transformant with W303-1B. After sporulation and tetrad dissection a spore with the desired genotype was obtained. Strains designated VMY in the text contain mutations that cause glucose-insensitive *ADH2* expression and are derived from either JSY24 (series denoted with "**a**" following the mutant isolation number) or VBY20 (series denoted with "α" following the mutant isolation number). The double mutants *vps35Δsnf1Δ::kanmx*, *cdc10-117***a***snf1Δ::kanmx*, and *adr1Δ::kanmx VMYx* shown in Figure 7 and supplemental Figure 1 (http://www.genetics.org/supplemental/) were the products of random spore analysis from diploids made from crossing NKY89α × VMY48**a** (*vps35*), NKY89α × VMY117**a** (*cdc10*), and NKY66α × VMY**a** mutants. The random spores were not saved. Knockouts were made using a kanamycin or nourseothricin deletion cassettes (GULDENER *et al.* 1996). The oligonucleotide sequences used are listed in supplemental Table 1 at http://www.genetics.org/supplemental/.

Yeast genomic libraries of plasmids based on YCp50 or pRS316 (*CEN4-URA3*) were used for cloning by complementation of function. Yeast plasmids containing septin genes were provided by J. Pringle and the *FAB1* kinase-dead plasmid was provided by S. Emr.

Growth of yeast cultures: Yeast strains were grown in YPD or synthetic medium prepared according to standard methods (GUTHRIE and FINK 2002). For maintaining glucose repression YP medium contained 8% glucose (YP-high-glucose) and cell density was kept under $OD_{600} = 0.8$. X-gal-8% glucose plates used to visualize constitutive expression of *ADH2/lacZ* were prepared from synthetic medium containing 40 μg/ml 5-bromo-4-chloro-3-idolyl-beta-D-galactopyranoside (X-gal), pH 7.0 (GUARENTE 1983). Plates containing 5′-fluoro-orotic (5′-FOA) acid were prepared as described (BOEKE *et al.* 1987) and contained 0.1 mg/ml 5′-FOA. Plates containing 1 μg/ml antimycin A were made to select for *adh1* mutant cells that have an *ADH2*-constitutive, antimycin A (At)-resistant phenotype (WILLIAMSON *et al.* 1980).

Mutant isolation: Strains JSY20, JSY21, JSY24, and VBY20 were treated with 3% ethyl methanesulfonate (EMS) to give 50–60% survival. Cells were plated and incubated at 30° or 25° for 2–3 days and then replica plated onto X-gal-8% glucose plates. Blue colonies on X-gal-high-glucose plates, indicating constitutive activity of an *ADH2/lacZ* reporter gene under conditions that are repressive for *ADH2* expression, were picked, restreaked, and retested. Approximately 60,000, 60,000, 50,000, and 30,000 colonies derived from JSY24, VBY20, JSY20, and JSY21, respectively, were tested. The mutant strains derived from strain JSY24 are designated VMY*n***a**, where *n* is the mutant isolation number and **a** refers to *MAT***a**. Similarly, VMY*n*α strains are derived from VBY20.

Genetic analysis of mutants: Complementation tests were performed by pairwise matings of all mutants. Diploids were

TABLE 1

Saccharomyces cerevisiae **strains**

Strains	Genotype	Source
JSY24	*MAT**a** adh3 ura3 leu2::(pRS315-ADR1(LEU2))X3 ADH2::YIpADH2/lacZ (TRP1)*	SLOAN *et al.* (1999)
JSY21	*MAT**a** adh3 ura3 leu2::pRS315-ADR1 (LEU2) ADH2::YIpADH2/lacZ (TRP1)*	SLOAN *et al.* (1999)
JSY20	*MAT**a** adh3 ura3 adr1Δ1::LEU2 ADH2::YIpADH2/lacZ(TRP1)*	SLOAN *et al.* (1999)
W303-1B	*MATα ade2 cam1-100 his3-11,15 leu2-13, 112 trp1-1 ura3-1*	Yeast stock center
NKY66	W303-1B with *Δadr1::kanmx ADH2::YIpADH2/lacZ (TRP1)*	This work
VBY20	*MATα adh3 ura3 his3 leu2::(pRS315-ADR1)X3 ADH2::YIpADH2/lacZ(TRP1)ADH2::* *YIpADH2/GFP (URA3)*	This work
KT1640	*MATα glc7-127 his3 leu2 ura3*	K. Tatchell
KDY87	*MATα his3 reg1Δ::LEU2 ura3 trp1*	K. Dombek
SSH35	*MATα adh1 adh3 adr1Δ1::LEU2 trp1 leu2 ura3*	KARNITZ *et al.* (1992)
LK13	SSH35 with *ADH2-10*	This work
LK13ΔS	LK13 with *snf1::URA3*	This work
S54	SSH35 with *adr7-1*	KARNITZ *et al.* (1992)
S54ΔS	S54 with *snf1::URA3*	KARNITZ *et al.* (1992)
LK14	S54 with *ADH2-10*	This work
LK14ΔS	LK14 with *snf1::URA3*	This work
CTY-TY10	JSY24 with *FAB1-HA₃-kanmx*	This work
DYY2 (931)	VBY20 *Δpep4::nat1*	This work
DYY4 (933)	VBY20 *Δshs1::nat1*	This work
DYY7 (936)	VBY20 *Δvac7::nat1*	This work
DYY14 (943)	VBY20 *Δvps34::nat1*	This work
DYY16 (945)	JSY24 *Δsnf1::kanmx*	This work
DYY 18 (947)	JSY24 *Δelm1::nat1*	This work
DYY19 (948)	VBY20 *Δelm1::nat1*	This work
DYY24 (953)	VBY20 *Δreg1::nat1*	This work
DYY28 (957)	VBY20 *Δcla4::nat1*	This work
DYY31 (959)	VBY20 *Δ420-Celm1::kanmx*	This work
NKY75	VBY20 *Δcdc10::nat1*	This work
NKY76	VBY20 *Δfab1::nat1*	This work
NKY89	VBY20 *Δsnf1::nat1*	This work
NKY99	JSY24 *Δsnf1::kanmx Δreg1::nat1*	This work
NKY77	VBY20 *Δvps35::nat1*	This work
EAY9	*MAT**a** Δfab1::nat1 Δsnf1::kanmx HIS3* (other markers the same as VBY20)	This work
EAY3 (994)	*MAT**a** Δvps34::nat1 Δsnf1::kanmx* (other markers the same as VBY20)	This work

selected by their Ura⁺His⁺ prototrophy and complementation was assessed by colony color on X-gal-8% glucose plates. Representatives from each of the complementation groups were crossed to congenic strains of the opposite mating type to determine whether the constitutive phenotype was due to a single mutation. After sporulation and tetrad dissection spore colonies were replica-plated onto X-gal-8% glucose plates and segregation of the blue/white color among spore colonies from a tetrad was determined.

Cloning: *ADR22 (complementation group XII)—FAB1:* Strain VMY115**a** (ts, blue on X-gal-8% glucose) was transformed with a yeast genomic library based on pRS316 (*CEN4/URA3*) at 30°. Ura⁺ transformants were replica plated onto YP-8% glucose for growth at 35° and onto X-gal-8% glucose plates at 30°. Twenty-five white, temperature-resistant colonies were streaked onto YPD before further analysis. Selected candidates were streaked from the YPD plates onto medium containing 5′-FOA. Twenty-two candidates that gave some 5′-FOA-resistant colonies, indicating the presence of a nonintegrated Ura⁺ plasmid, were examined further at 35° and on X-gal-8% glucose plates. The library plasmids were recovered from the candidates that exhibited plasmid-dependent complementation of the mutant phenotype, and their ability to complement the original mutant strain was confirmed by retransformation with the purified plasmid. The same plasmids also comple-

mented strain VMY89α, another mutant in complementation group *ADR22*. Restriction analysis revealed that the complementing plasmids were of three types, designated 115pI, 115pII, and 115pIII. The plasmid inserts were sequenced using the primers YCP50-B1 and YCP50-B2 in the University of Washington Sequencing Facility. The common portion of these three overlapping genomic regions has only one ORF, the *FAB1* gene. Plasmids 115pII and 115pIII differ only in the orientation of the genomic fragment present in the vector. The genomic fragment in these plasmids contains a 5′-truncated fragment of the *FAB1* gene. A similar fragment complements an *FAB1* deletion mutant (YAMAMOTO *et al.* 1995). 115pI contained an intact *FAB1* gene. Since the only genomic region in common in these three plasmids contains *FAB1*, or a functional version of *FAB1*, it is likely that *FAB1* corresponds to *ADR22*. This interpretation was confirmed as described in RESULTS.

ADR11 (complementation group I)—VPS35: A gene representing complementation group I (*ADR11*) was cloned by complementing the blue-color phenotype on X-gal-8% plates using a yeast genomic library carried on a CEN plasmid. About 8000 Ura⁺ transformants of strains VMY33**a** and VMY48**a** were selected from each transformation and replica plated onto X-gal-8% glucose plates. Colonies that were white, indicating complementation, were restreaked. Candidate colonies were

selected for further study if they were blue after losing the library plasmid by selection on FOA plates.

Six of eight independently isolated plasmids from positive transformants of VMY33**a** had identical restriction maps with three restriction enzymes and two had slightly different maps. The two types of plasmids were sequenced from both sides of the insert. The majority of plasmids contained a region of chromosome X that included two intact ORFs, *VPS35* and *INO1*. A second class of complementing plasmids contained almost the same chromosomal region but the 5′ end of the *INO1* gene was missing. Candidate transformants of VMY48**a** strain yielded two library plasmids. When sequenced, they appeared to contain the same chromosomal region as the majority of complementing plasmids from the transformation of VMY33**a**, including the *VPS35* and *INO1* genes. The identification of the mutation in VMY48**a** was determined by sequencing the entire gene using primers listed in supplemental Table 1 (http://www.genetics.org/supplemental/).

ADR18 (complementation group VIII)—CDC10: Strain VMY117**a** was transformed with a yeast genomic library based on pRS316. Approximately 20,000 transformants were plated on SD-ura plates, allowed to recover at room temperature overnight, and then transferred to 37°. After 2 days of incubation four temperature-resistant colonies were isolated. The ts^R, Ura$^+$ transformants were β-galactosidase negative, indicating that the constitutive *ADH2/lacZ* expression in the parent was complemented by the library plasmids. The abnormal cell morphology of VMY117**a**, long chains of cells with incomplete cytokinesis, was completely suppressed by the library plasmids. Restriction analyses suggested that each transformant contained one or the other of two types of plasmids with overlapping genomic fragments. Retransformation confirmed that the temperature-resistant phenotype and reduced β-galactosidase activity were plasmid dependent. Sequencing with T3 and T7 primers revealed that both types of plasmids contained DNA fragments derived from chromosome III (nucleotides 109224–121326). Only one gene in this region, *CDC10*, has mutant phenotypes of temperature sensitivity and incomplete cytokinesis that were observed in strain VMY117**a** (Hartwell 1971; Giaever *et al.* 2002). *CDC10* encodes one of the yeast septins, a family of proteins that form 10-nm fibers involved in cytokinesis-related functions. Nucleotides 109224–116148 of the insert in the library plasmid pLTR(117) were removed by digestion with *Xba*I to create plasmid pLTR(117)X (containing nucleotides 116149–121326, which includes all of *CDC10*). Transformation of strain VMY117**a** with pLTR(117)X allowed growth at 37° and completely suppressed the abnormal cell morphology. The *CDC10* allele in strain VMY117**a** was sequenced on both strands and a single G-to-A base-pair substitution was found at position 131 in the ORF confirming that *ADR18* is allelic to *CDC10*.

ADR7 (mutant13a)—(MOT1): Mutant 13**a** failed to complement the temperature sensitivity of an *adr7* mutant isolated in an earlier screen (Karnitz *et al.*, 1992). To clone the *adr7* mutant, ∼30,000 colonies of strain S54 (*adh1 adr7-1*) transformed with a yeast genomic library based on Ycp50 were screened for growth at 37°. Temperature-resistant colonies were tested for complementation of a second mutant phenotype, resistance to the respiratory inhibitor Antimycin A (At). The At^R phenotype is a consequence of constitutive expression of *ADH2* in strain S54 (Karnitz *et al.* 1992). Seven of the temperature-resistant colonies were also At^s and thus contained potential *ADR7*-complementing plasmids. Plasmids from the two At^s Ts$^+$ colonies complemented the *adr7-1* mutant phenotype (ts and At^R) of strain S54. Sequencing revealed that they contained the same 14-kbp chromosome XVI fragment. Deletion of an internal 3.6-kbp *Bam*HI fragment eliminated the complementing activity of the plasmid.

Subsequent subcloning and sequencing of that region revealed that it contains the *MOT1* gene. When strain VMY13**a** was transformed with the cloned library plasmid containing *MOT1*, both the ts and *ADH2/lacZ* constitutive phenotypes were complemented by the *MOT1* library plasmid, suggesting that the *adr7-1* mutation and the mutation in strain VMY13**a** are allelic (V. Voronkova, unpublished data).

To test if *MOT1* and *ADR7* are allelic, the *adr7*-1 mutation was mapped to the *MOT1* locus by integrative transformation and subsequent genetic analysis. A 5′ portion of *MOT1* including the promoter but lacking the essential helicase domain was subcloned into pRS306, an integrative *URA3* vector, to produce pRS306-SSII. Strain S54 (*adr7-1*) was transformed with pRS306-SSII linearized by *Nhe*I digestion. The resulting strain was crossed to SSH46, a *MOT1* wild-type strain. Diploids were selected and sporulated. The phenotypes of the resulting spores were analyzed (Ura$^+$/Ura$^-$, At^R/At^s, growth at 37°). In 18 full tetrads, the Ura$^+$ phenotype always cosegregated with the *adr7* phenotype, indicating that *ADR7* and *MOT1* are allelic or very closely linked (V. Voronkova, unpublished data).

Enzyme assays: β-Galactosidase assays were performed on permeabilized cells (Guarente 1983). Activities are expressed in Miller units. Secreted invertase activity was assayed in whole cells (Jiang and Carlson 1997). ADH assays were performed on whole cell extracts and by *in situ* staining of polyacrylamide gels (Dombek *et al.* 1993).

Protein extracts and Western blotting: Denatured whole-cell extracts were prepared as described previously (Dombek *et al.* 1993) or as in Kushnirov (2000). Proteins were separated by SDS–PAGE, transferred to a nylon membrane, and analyzed by immunoblotting using polyclonal rabbit antibodies raised against amino acids 335–740 of Adr1 and IR-labeled secondary antibodies (Rockland). Visualization and quantitation were performed on an Odyssey infrared imaging system (Li-Cor Biosciences) according to manufacturer's directions.

RESULTS

Constitutive *ADH2* expression in *ADR1* multicopy strains lacking *REG1* or Glc7 protein phosphatase activity: In preparation for using multicopy *ADR1* strains in a genetic screen for constitutive *ADH2* expression, we tested their ability to detect known mutations in *ADH2* regulation by deleting *REG1* and *SNF1* in VBY20. *GLC7* was deleted in the presence of a ts allele of *GLC7* carried on a plasmid (Dombek *et al.* 1993, 1999). The multicopy strains reveal the constitutive phenotypes of the *reg1* and *glc7* mutants by both an in-gel activity assay for endogenous *ADH2* expression and by an *ADH2/lacZ* reporter assay. Snf1 is required for derepression in the multicopy *ADR1* strain (Figure 1) and for constitutive expression in the multicopy *ADR1, reg1*-deletion strain (N. Kacherovsky, unpublished data). Thus, the multicopy *ADR1* strain is an appropriate starting point for screening for regulatory mutations affecting *ADH2* repression.

Mutant screen: Mutagenesis of JSY24 (*MAT***a**) and VBY20 (*MAT*α), both of which contain three extra copies of *ADR1* integrated at the *LEU2* locus, yielded 191 mutants that exhibited constitutive expression of the integrated *ADH2/lacZ* reporter. Strains with zero (JSY20) or one (JSY21) copy of *ADR1* yielded one and
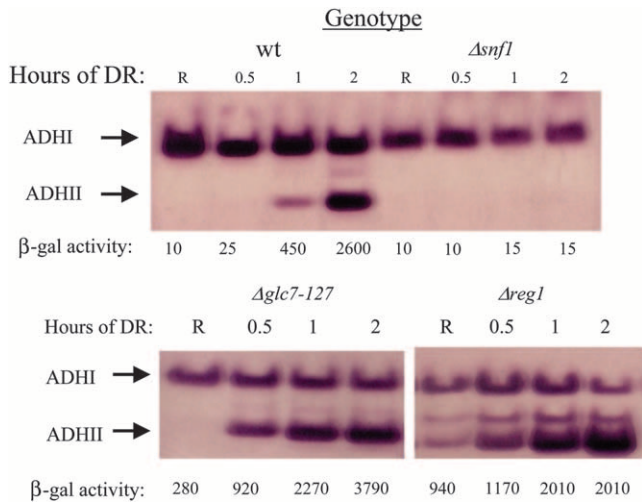
FIGURE 1.—Constitutive *ADH2* and *ADH2/lacZ* expression in multicopy *ADR1* strains with mutations in regulatory genes. In-gel ADH activity assays of extracts prepared from strain JSY24, Δ*snf1*, Δ*reg1*, and Δ*glc7* (YCp*GLC7-127*) were performed as described in MATERIALS AND METHODS. R indicates extracts prepared from repressed cultures grown at 30°. The other time points represent hours after shifting the cells to derepressing (DR) medium (YP with 0.05% glucose). The numbers below each lane represent the β-galactosidase activities in Miller units. The band between ADHI and ADHII also represents ADHII expression, since it is a heterodimer of the two enzymes.

two mutants, respectively, when the same number of surviving cells was plated. The dramatic difference in the number of mutants isolated from strains with zero (or one) and three integrated copies of *ADR1* confirms the importance of performing the mutant isolation in a strain that expresses Adr1 at derepressed levels. Presumably, the additional amount of Adr1 magnifies the effect of a mutation that allows expression from the *ADH2* promoter under repressed conditions. Thus, by satisfying the requirement for the positive activator, we can more readily identify genes that play a negative role in *ADH2* expression. The constitutive *ADH2/lacZ* expression in the mutant strains could be due either to *cis*-acting mutations located in the promoter region of the *ADH2/lacZ* reporter gene or to *trans*-acting mutations that affect its expression. Expression of the chromosomal *ADH2* locus was assayed to distinguish between these possibilities since only *trans*-acting mutations would affect its expression. The majority of the mutants exhibited ADHII activity after growth under repressed conditions, as revealed by in-gel activity assays (V. VORONKONVA, unpublished data). The mutants that did not show detectable ADHII activity were not studied further (~10% of mutants). Thus, most of the mutations conferring constitutive activity on an *ADH2/lacZ* reporter gene also cause constitutive expression of the chromosomal *ADH2* locus and are therefore *trans*-acting.

Genetic analysis: To determine whether the mutants are recessive or dominant they were backcrossed to a

congenic wild-type strain and the constitutive *ADH2/lacZ* phenotype was determined. Most of the diploids were white, indicating a recessive mutation, but several of them were blue (dominant). Among 101 *MAT***a** mutants 93 are recessive and 8 are dominant. Among 90 *MAT*α mutants 79 are recessive and 11 are dominant.

Complementation tests were performed by pairwise matings of all recessive mutants. The diploids were selected and examined for *ADH2/lacZ* constitutive activity on X-gal-8% glucose plates. One-third of the *MAT***a** and most of the *MAT*α mutants belong to 1 of 12 complementation groups, I–XII (Table 2). We named the 12 genes represented by these complementation groups *ADR11–ADR22* (*a*lcohol *d*ehydrogenase *r*egulator 11–22). The mutants that do not fall into any of the 12 complementation groups could harbor multiple mutations, represent additional genes affecting *ADH2* repression, or harbor weak alleles that fail to exhibit a constitutive *ADH2* phenotype in a diploid strain. All mutants were also tested for complementation of mutations in *REG1*, *GLC7*, *ADR7*, *ADR8*, and *ADR9* since mutations in these genes cause constitutive *ADH2* expression (KARNITZ *et al.* 1992; DOMBEK *et al.* 1993, 1999). VMY13**a** and VMY78**a**, which did not belong to one of the complementation groups containing multiple alleles, failed to complement strains containing mutations in *ADR7* and *ADR8*, respectively, suggesting that they contain mutant alleles of those genes.

Several cases of nonallelic noncomplementation were observed, suggesting that some complementation groups contain mutant alleles from different genes. For example, mutant VMY37**a** fails to complement mutants VMY62α and VMY1α in complementation group I and also fails to complement mutant VMY44α in complementation group IX. To determine the true complementation group for mutant VMY37**a**, diploids derived from crosses of VMY37**a** with VMY62α and with VMY44α were subjected to tetrad analysis. If the two parents harbor a mutation in the same gene, then all four spores will inherit a mutation in the same gene and will have a mutant phenotype. A 4:0 segregation of blue/white spores was observed for diploids derived from crossing VMY37**a** with a member of complementation group IX, VMY44α, but not from a cross with VMY62α, a mutant from complementation group I. This result indicates that mutant VMY37**a** belongs to complementation group IX and shows nonallelic noncomplementation with mutants in complementation group I.

Because several members of complementation group I showed aberrant complementation behavior, we confirmed that assignment in the following manner. *MAT*α derivatives of VMY48**a** (harboring a mutation in complementation group I) and two mutants that do not belong to any complementation group, VMY25**a** and VMY42**a**, were obtained from a backcross to the parental strain and were crossed to the whole set of the *MAT***a** mutants. The diploids obtained were tested for complementation

| Complementation group | Gene | Allele | | Additional phenotypes |
		*MAT*a	*MAT*α	
I	*ADR11*	*33,[a] 38, 48,[a,b] 109, 114,[a] 37[a,b]*	*1, 30, 32, 62*	Elevated *ADH2* Expression at 25°C
II	*ADR12*	*17[a]*	*5, 9, 59, 89[a,b]*	Abn
III	*ADR13*	*70, 112*	*19, 35, 85*	Spo⁻
IV	*ADR14*	*103, 119[a]*	*6, 10*	Abn
V	*ADR15*	*3,[b] 15*	*52*	Spo⁻
VI	*ADR16*	*3,[b] 63,[a] 101[a]*	*25*	
VII	*ADR17*	*110*	*20, 66*	Spo⁻ Inv[C]
VIII	*ADR18*	*53, 117,[a] 141*	*49*	ts, hs, Abn Inv[C]
IX	*ADR19*	*37,[a,b] 59, 127, 128*	*44*	ts, hs, Inv[C]
X	*ADR20*	*16, 43*	*97, 102*	
XI	*ADR21*	*44, 48,[a,b] 105, 115[a,b]*	*79*	ts
XII	*ADR22*	*115[a,b]*	*89[a,b]*	ts

Additional mutants that did not fall into any of the complementation groups are not shown and were not studied further except for two that failed to complement known genes. They are mutant 13**a**, a temperature-sensitive mutant that failed to complement mutations in *ADR7*, and mutant 78**a**, which failed to complement mutations in *ADR8*.

Spo⁻, sporulation deficiency in heterozygous diploids; Abn, abnormal cell morphology; Inv[C], constitutive invertase activity; ts, temperature sensitive; hs, heat-shock sensitive.

[a] Phenotype is due to a single gene.

[b] Shows nonallelic noncomplementation. That is, it complements mutants in more than one complementation group. For example, VMY89α fails to complement mutants in both complementation group II and complementation group XII. In the case of VMY89α and VMY115**a**, the true complementation group is XII; for VMY48**a**, the true complementation group is I; and for VMY37**a**, the true complementation group is IX, as described in the text. The true complementation group for VMY3**a** was not determined.

of the mutant *ADH2/lacZ* phenotype. The *MAT*α derivative of VMY48**a** failed to complement all *MAT*a members of complementation group I but complemented all other *MAT*a mutants. The *MAT*α derivatives of VMY25**a** and VMY42**a** failed to complement only the original mutants. These results suggest that the observation of nonallelic noncomplementation is not due to incorrect assignment of the mutations to their complementation groups. Although the importance of these observations is unclear, it suggests that the genes represented by these complementation groups carry out functionally related processes affecting *ADH2* repression.

Constitutive chromosomal *ADH2* expression: To confirm that the mutations in the defined complementation groups are *trans*-acting and thus affect the expression of the chromosomal *ADH2* gene we per-

formed in-gel ADH activity assays for two mutants in each complementation group. The mutants analyzed each contain a single mutation that affects *ADH2/lacZ* expression as shown by tetrad analysis. Mutants from each complementation group exhibit different but consistent levels of constitutive ADHII activity (Figure 2).

Growth phenotypes associated with *adr11-adr22* mutants: We tested the mutants for heat and cold sensitivity, heat-shock sensitivity, abnormal cell morphology, and enhanced β-galactosidase activity at 25° or 35° (data summarized in Table 2 for mutants in complementation groups I–XII). Five mutants, representing 4 complementation groups (VIII, IX, XI, XII), as well as five mutants not belonging to one of the 12 groups, are sensitive to elevated temperature (ts), but none are cold sensitive. The two ts mutants in complementation groups
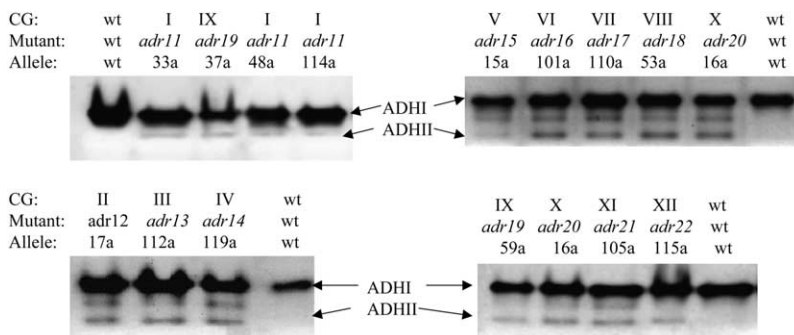

FIGURE 2.—Constitutive ADHII activity of representatives from complementation groups I–XII (*adr11-adr22*). In-gel ADH activity assays were performed as described in MATERIALS AND METHODS. Cell extracts were prepared from cultures grown in YP-8% glucose to an A600 ∼1. JSY24 is the wild-type parental strain. The band between ADHI and II also represents ADHII expression since it is a heterodimer of the two enzymes. See Table 2 for a complete description of the mutant phenotypes.
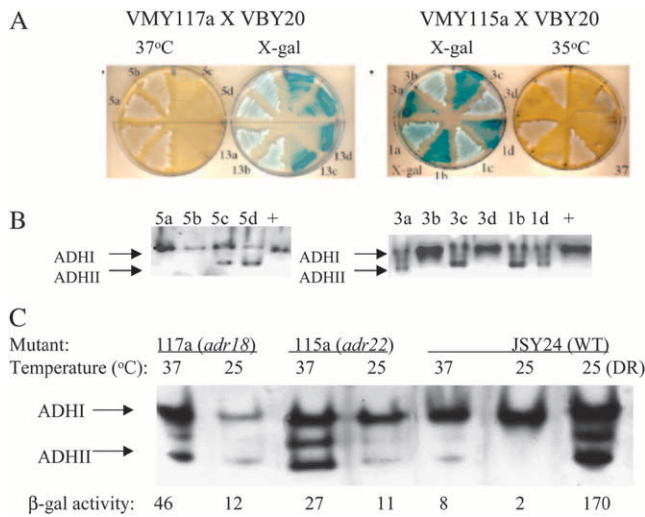
FIGURE 3.—A single mutation causes temperature sensitivity and constitutive *ADH2* expression in mutants VMY115**a** and VMY117**a**. Spore colonies from dissected tetrads arising from a cross of the mutant strain VMY117**a** (tetrads 5 and 13) and VMY115**a** (tetrads 1 and 3) with VBY20 were tested for β-galactosidase activity on X-gal-8% glucose plates and for temperature sensitivity (A). ADH activity was assayed using an in-gel assay of selected spore colonies or wild type (+ lane) (B). Temperature sensitivity of *ADH2* expression in VMY117**a** and VMY115**a** was assayed by in-gel assays of ADH activity and *ADH2/lacZ* expression at 25° and 2 hr after a shift to 37°. The ADH and β-galactosidase activity assays were performed on repressed cultures. JSY24 is the parental strain for VMY115**a** and VMY117**a**.

TABLE 3

Invertase and *ADH2*/β-galactosidase activities in *adr* mutant strains

Gene	Alleles	Invertase activity		β-Galactosidase activity	
		R	DR	R	DR
ADR11	33**a**	2	260	13	320
	114**a**	3	260	11	350
ADR12	17**a**	8	270	16	310
ADR13	70**a**	3	220	20	270
	112**a**	5	240	28	330
ADR14	119**a**	2	250	18	300
	103**a**	5	230	16	280
ADR15	3**a**	3	250	15	330
	15**a**	5	280	12	350
ADR16	63**a**	8	250	16	320
	101**a**	6	280	18	350
ADR17	110**a**	12	270	14	360
ADR18	53**a**	10	210	24	300
	117**a**	24	400	20	360
ADR19	37**a**	35	400	26	360
	127**a**	12	280	18	320
ADR20	16**a**	6	280	14	320
	43**a**	3	250	14	300
ADR21	105**a**	4	250	14	300
	44**a**	6	270	15	350
ADR22	115**a**	4	320	19	370
Wild type (JSY24)		3	280	2	350

Enzyme activities were determined as described in MATERIALS AND METHODS. The values represent the average of three determinations. The standard deviation for each value was ~10%.

R, repressed; DR, derepressed.

VIII and IX are heat-shock sensitive as well. ts mutant VMY117**a** in complementation group VIII showed a pronounced cytokinesis defect even at the permissive growth temperature. All members of complementation group I displayed enhanced blue color at 25° on 8% glucose X-gal plates compared to wild type, suggesting a cold-sensitive step in *ADH2* repression in these mutants. The ts mutants are particularly interesting because they indicate that an essential gene plays a role in repressing *ADH2* expression. To determine whether the ts and the constitutive *ADH2* phenotypes are due to the same mutation, the strains bearing ts alleles were back-crossed to the appropriate parental strain. The heterozygous diploids were sporulated and the distribution of phenotypes among spore colonies was analyzed. The constitutive β-galactosidase activity, ADHII activity on glucose measured by in-gel assay, and temperature sensitivity segregated 2:2 and cosegregated with each other for mutants VMY115**a** and VMY117**a** (Figure 3, A and B). Mutants VMY89α and VMY111**a** behaved similarly (V. VORONKOVA, unpublished data). Thus, these four strains harbor mutations in a single gene that is essential and is involved in glucose repression of *ADH2* expression. A similar analysis was performed on 12 other mutants. In six cases the constitutive phenotype appeared to be due to more than one mutation, suggesting

that some of the mutants harbor multiple lesions that are responsible for their constitutive activity.

If a single mutant gene is responsible for impaired *ADH2* glucose repression and ts growth phenotype, then at the nonpermissive temperature the mutant protein should not only fail to support growth but also fail to sustain *ADH2* repression. To test this we assayed *ADH2* constitutive activity at the nonpermissive temperature. Mutants were grown at the permissive (25°) temperature and then shifted to 37°. Cells were collected and β-galactosidase and ADH II activities were determined. Figure 3C shows that at the nonpermissive temperature mutants VMY115**a** and VMY117**a** exhibited increased β-galactosidase and ADHII activities, consistent with the interpretation that a single mutation is responsible for both phenotypes.

Invertase and *ADH2* expression in *adr* mutants: We measured secreted invertase activity of two members of each complementation group after growth on glucose to determine if the mutants affect other glucose-repressed genes. Strains containing mutations in three complementation groups, *ADR17, ADR18,* and *ADR19,* have elevated levels of secreted invertase activity when grown under repressed conditions (Table 3). The highest invertase activity is exhibited by strains containing

either one of two temperature-sensitive mutations, 117**a** (*adr18*, 8-fold wild type) or 37**a** (*adr19*, 11-fold wild type).

β-Galactosidase activity of the same mutants was determined in the same preparations to have a quantitative measure of constitutive expression of the *ADH2/lacZ* reporter (Table 3). The constitutive activity in the mutant cells is 5–14 times higher than the activity under repressed conditions of wild-type cells. Under derepressed conditions the β-galactosidase activities are close to that of the wild-type strain as measured by an endpoint assay. The constitutive activities in the mutant strains represent 3–10% of the activity after derepression, significantly lower than the activity present in the absence of Reg1 (Figure 1) suggesting that these mutations and a *reg1* deletion do not disrupt the same pathway of repression.

ADR1-dependence of constitutive ADH2 expression: The large number of constitutive mutants isolated in the multicopy *ADR1* strains compared to strains with a single copy of *ADR1* suggests that the constitutive *ADH2* expression is *ADR1* dependent. This prediction was tested by analyzing spore colonies grown from tetrads derived from crosses of one mutant from each complementation group to a congenic wild-type strain of the opposite mating type in which *ADR1* had been deleted and replaced by a kanMX cassette. The different alleles of *ADR1* could be identified from their phenotypes on plates (*adr1* null: Leu⁻, Kan^R; single copy: Kan^S, Leu⁻; or multicopy: Leu⁺, Kan^S, or Kan^R). Constitutive *ADH2/lacZ* expression was monitored on X-gal plates. Because the mutation could not be followed directly, its presence or absence was inferred from the color on indicator plates. Thus, if all *ADR1*-null (Leu⁻ Kan^R) spore colonies were white or light blue on X-gal plates, and some multicopy (Leu⁺) *ADR1* spore colonies were dark blue, the phenotype was judged to be *ADR1* dependent. The level of Adr1 in suspected mutant spore colonies was confirmed by Western blotting for Adr1. On the basis of these criteria, the constitutive *ADH2* expression in all of the mutants is *ADR1* dependent, although some, such as VMY89α, showed a stronger dependence on *ADR1* copy number than others, such as VMY49α (D. Yu, unpublished data, and supplemental Figure 1 at http://www.genetics.org/supplemental/).

Cloning and identifying ADR11, ADR18, ADR22, and ADR7: *Complementation group XII—ADR22 (FAB1):* Complementation group XII (*ADR22*) consists of two ts mutants, 115**a** and 89α, that exhibit impaired glucose repression of *ADH2* but not *SUC2* expression. Thus, *ADR22* is an essential gene that is involved in a pathway of glucose repression that affects *ADH2* but not *SUC2* expression. This complementation group is also interesting because both 115**a** and 89α exhibit nonallelic noncomplementation with mutants in other complementation groups (Table 2).

ADR22 was cloned by complementing the constitutive *ADH2* expression and ts mutant phenotypes using a low-copy yeast genomic library (MATERIALS AND METHODS). The common portion of the cloned genomic regions have only one complete or nearly complete ORF and it encodes the *FAB1* gene encoding 1-phosphatidylinositol-3′-phosphate-5′-kinase [PtdIns (3′)-P-(5′)-kinase] (YAMAMOTO *et al.* 1995; BONANGELINO *et al.* 2002; GARY *et al.* 2002).

The cloned *FAB1* gene complemented all mutant phenotypes of VMY89α and VMY115**a**, suggesting that *FAB1* corresponds to *ADR22*. An enlarged vacuole is a phenotype previously noted for conditional *FAB1* mutants (YAMAMOTO *et al.* 1995). The greatly enlarged single vacuole in the 115**a** and 89α mutants is restored to wild-type size and shape by *FAB1*-containing plasmids (V. VORONKOVA, unpublished data). A deletion of *FAB1* has a ts phenotype (YAMAMOTO *et al.* 1995) consistent with the phenotype of strains VMY115**a** and VMY89α and suggesting that these mutations represent the null *FAB1* phenotype.

A *FAB1* mutation in VMY115**a** was confirmed in three ways. First, when a diploid *VMY115**a** fab1*ᵗˢ*/FAB1:kanMX*-tag strain was sporulated, nine of nine full tetrads segregated 2:2 for G418^R:ts (C. TACHIBANA, unpublished data). Since the ts character and *ADH2* constitutivity are genetically linked (Figure 3), strains VMY115**a** and VMY89α most likely contain mutant alleles of *FAB1*. Second, the entire *FAB1* ORF was sequenced in strains VMY115**a** and VMY89α. A nonsense mutation, CAA to TAA, corresponding to Gln1686, is present in both strains causing loss of the C-terminal kinase domain. This mutation is sufficient to explain the temperature sensitivity of strains VMY115**a** and VMY89α since a kinase-dead mutant of *FAB1* is temperature sensitive (YAMAMOTO *et al.* 1995). Third, we tested a kinase-dead mutant for constitutive *ADH2*/lacZ expression by introducing a plasmid carrying a *fab1-kd* allele into VBY20Δ*fab1*. The kinase-dead allele, a substitution of Asp2134 by Arg in the kinase catalytic domain, failed to complement the constitutive activity and temperature sensitivity, whereas the wild-type *FAB1* gene carried on a plasmid complemented both phenotypes (Figure 4) indicating that Fab1 kinase activity is required to maintain glucose repression of *ADH2* expression.

Strains VMY115**a** and VMY89α (*ADR22*) failed to complement strains containing mutations in *ADR12* and *ADR21*. These mutants were not complemented with a plasmid containing *FAB1*, confirming that *ADR12* and *ADR21* show nonallelic noncomplementation with *FAB1*.

Fab1 phosphorylates PtdIns-3′-phosphate, the product of the reaction catalyzed by the PtdIns-3′-kinase Vps34. If PtdIns-3′,5′-phosphate is important in glucose repression, as suggested by the constitutive activity of *ADH2* in a *fab1* mutant, constitutive *ADH2* expression would be expected in a *vps34* mutant strain. Constitutive *ADH2* expression was indeed observed in the *ADR1* multicopy strain VBY20 with a *vps34::kanMX* allele, and, like the *fab1* mutants, the strain is temperature sensitive
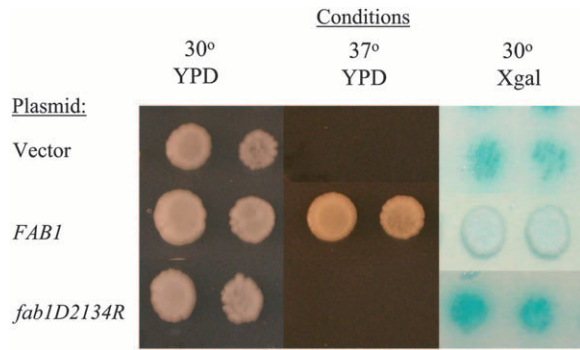
FIGURE 4.—Repression of *ADH2* expression requires the kinase activity of Fab1. VBY20Δ*fab1* was transformed with *URA3*-CEN plasmids pRS316, p*FAB1(115pI)*, or p*fab1D2134R*. Transformants were grown to log-phase in YPD (8% glucose), serially diluted, and spotted onto YPD plates at 30° and 37° and onto X-gal-8% glucose plate at 30°. Two dilutions are shown.

for growth (Figure 5A). The strain with a deletion of *VPS34* grew very poorly on the X-gal plates, indicative of a high level of constitutive *ADH2/lacZ* expression. As with the strain with a mutation in *FAB1*, the constitutive *ADH2/lacZ* expression was elevated at 30° and 33° compared to 22°. This result indicates that PtdIns-3′-phosphate is important in glucose repression, presumably as a precursor to PtdIns-3′,5′-phosphate. *VAC7* encodes a protein that, like Fab1 and Vps34, is implicated in vacuolar biogenesis (GARY *et al.* 2002). However, deleting *VAC7* did not cause constitutive *ADH2/lacZ* expression, indicating that Vac7 does not play a role in *ADH2* repression (Figure 5A). A defect in general vacuolar function is not the source of constitutive *ADH2/lacZ* expression because deletion of *PEP4*, a gene encoding the major processing protease in the vacuole, did not cause constitutive *ADH2* expression (Figure 5A). In summary, the results implicate PtdIns-3′,5′-phosphate in maintaining glucose repression of *ADH2* expression.

Complementation group I—ADR11(VPS35): Complementation group I (*ADR11*) is the largest of the 12 complementation groups and all nine members exhibit enhanced *ADH2/lacZ* expression at 25°. Since *ADR11* mutants do not have constitutive invertase activity, they

are unlikely to have mutations in genes previously known to affect glucose repression. Mutations in this group also display nonallelic noncomplementation with ts mutant 37**a** in *ADR19*.

ADR11 was cloned by complementing the *ADH2/lacZ* constitutive mutant phenotype of strains VMY33**a** and VMY48**a** (MATERIALS AND METHODS). The data in MATERIALS AND METHODS suggest that *VPS35* is the complementing gene on the isolated plasmids. *VPS35* was recovered in a genetic screen to isolate mutants deficient in sorting hydrolases from the Golgi to the vacuole (PARAVICINI *et al.* 1992). Vps35 is a part of the retromer complex and is responsible for cargo-selective activity (BURDA *et al.* 2002).

The entire *VPS35* ORF in all mutants in complementation group I was sequenced. A single missense mutation was found in strain VMY48**a**, changing Glu433 to Gly in the *VPS35* ORF. None of the other mutants contained a mutation in the *VPS35* ORF, demonstrating that the Glu433-to-Gly change in VMY48**a** is a mutation and not a strain-specific polymorphism. Since all of the mutants in this complementation group were complemented by VPS35 carried on the library plasmid (N. KACHEROVSKY, unpublished data), they could contain non-ORF mutations that affect expression of *VPS35*, or they could, like VMY37**a**, represent examples of nonallelic noncomplementation. However, strain VMY37**a**, which has a mutation in complementation group IX and shows nonallelic noncomplementation with mutants in complementation group I, was not complemented by *VPS35* carried on a plasmid.

Complementation group VIII—ADR18 (CDC10): The mutation in strain VMY117**a**, in complementation group VIII (*ADR18*), causes temperature sensitivity, a cytokinesis defect, and constitutive *ADH2* and *SUC2* expression. Library plasmids complementing the temperature sensitivity of VMY117**a** contained overlapping fragments that also complemented the constitutive *ADH2* expression and incomplete cytokinesis. *CDC10* was shown by subcloning to be the ORF responsible for complementation. Mutations in *CDC10* as well as a deletion of the *CDC10* ORF cause temperature-sensitive growth and
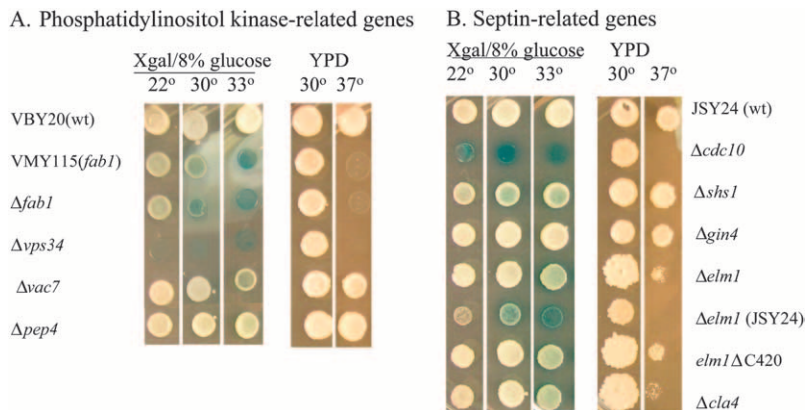

FIGURE 5.—Constitutive *ADH2/lacZ* expression in strains with mutations in phosphatidylinositol kinase- and septin-related genes. Growth and reporter tests were conducted by diluting log-phase yeast cultures and spotting serial dilutions onto YPD plates at 30° and 37° and onto X-gal-8% glucose plates that were incubated at 22°, 30°, and 33°. Only one dilution is shown. The strains used are described in the text and Table 1.
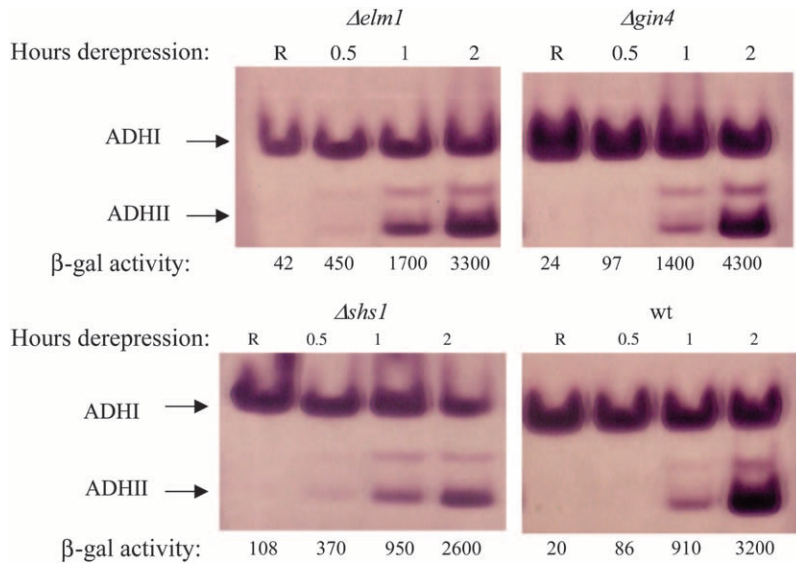
Figure 6.—*ADH2* expression in septin-related mutants *gin4*, *shs1*, and *elm1*. In-gel ADH activity assays were performed on extracts from strains with mutations that cause septin formation defects. R indicates extracts prepared from repressed cultures grown at 30°. The other time points represent hours after shifting the cells to derepressing medium (YP with 0.05% glucose). The numbers below each lane represent the β-galactosidase activities in Miller units.

incomplete cytokinesis (HARTWELL 1971; FRAZIER *et al.* 1998) suggesting that VMY117**a** contains a loss-of-function mutation in *CDC10*.

CDC10 encodes one of the yeast septins, a family of proteins that form 10-nm fibers involved in cytokinesis-related functions (LONGTINE and BI 2003). Proof that VMY117**a** is defective in *CDC10* was obtained by finding a single missense mutation, GGT to GAT, in *CDC10*. The mutation creates a Gly44-to-Asp substitution in a GTP-binding motif (FLESCHER *et al.* 1993). Mutations in the GTP-binding motif of the homologous yeast septin encoded by *CDC11* inhibit GTP binding but have only a modest phenotypic effect (CASAMAYOR and SNYDER 2003). The Gly44-to-Asp substitution in Cdc10 could have a more severe defect in GTP binding than related mutations in Cdc11. Alternatively, GTP binding and hydrolysis by Cdc10 may be more important for Cdc10 function than it is for Cdc11. A deletion of the *CDC10* locus was created by *nat1* (nourseothricin resistance) insertion in the *ADR1* multicopy strain VBY20. The resulting strain was ts, had a cytokinesis defect at 30°, and expressed *ADH2* constitutively (Figures 5B and 6, and N. KACHEROVSKY, unpublished data), consistent with the sequencing data indicating that a lesion in *CDC10* in strain VMY117**a** causes constitutive *ADH2* expression. Mutations in other nonessential septins (*SHS1*, *SPR3*, *SPR28*) as well as in genes functionally related to the septins (*CLA4*, *GIN4*, *ELM1*, *GAC1*) were tested for constitutive *ADH2/lacZ* expression in strain VBY20 after deletion using a *nat1* cassette. Strains lacking *SHS1*, encoding a nonessential septin homolog, and *ELM1*, encoding a protein kinase involved in septin assembly (SREENIVASAN and KELLOGG 1999) and also acting as a Snf1-activating kinase (HONG *et al.* 2003), had weak constitutive *ADH2/lacZ* expression that was elevated at 30° and 33° relative to 22°. In addition, at 33° constitutive *ADH2/lacZ* expression could be detected in the

strain deleted for *CLA4*. A strain in which the C-terminal inhibitory domain of Elm1 had been deleted had similar phenotypes. Deletion of *SPR3*, *SPR28*, *GIN4*, and *GAC1* did not cause constitutive *ADH2/lacZ* expression (Figures 5B and 6, and N. KACHEROVSKY, unpublished data). The constitutive activity of the *elm1* deletion allele, as well as temperature sensitivity, was more pronounced in JSY24, another multicopy *ADR1* strain (Figure 5B). Constitutive *ADH2* expression was also tested in *elm1*, *gin4*, or *shs1* deletion strains by measuring chromosomal *ADH2* expression with an in-gel activity assay and β-galactosidase activity from an integrated reporter. Although constitutive *ADH2* expression could not be detected by the in-gel assay, more rapid derepression of both *ADH2* and *ADH2/lacZ* was detected in *elmΔ* and *shs1Δ* strains compared to a wild-type strain or a strain with a *gin4* deletion. As predicted from the color phenotype of X-gal-8% glucose plates, the constitutive β-galactosidase activity was elevated in the *elmΔ* and *shs1Δ* strains (Figure 6). Two other strains in complementation group VIII, VMY53**a** and VMY49α, failed to be complemented by *CDC10* or the septins *CDC3*, *CDC11*, *CDC12*, or *SHS1* on a plasmid. Sequencing of the entire *CDC10* ORF in these strains did not reveal a mutation (N. KACHEROVSKY, unpublished data). These strains apparently show non-allelic noncomplementation with a strain with a mutation in *CDC10*. In summary, alteration of septin function or assembly can result in constitutive *ADH2* and *SUC2* expression.

SNF1-dependence of constitutive activity in strains containing mutations in *VPS35*, *FAB1*, *VPS34*, or *CDC10*: The *SNF1* dependence of constitutive *ADH2* expression was tested by deleting *SNF1* using a *kanMX* or *nat1* cassette in strains VMY48**a** (*VPS35*), VMY89α (*FAB1*), and VMY117**a** (*CDC10*). The *SNF1* dependence of *VPS34* was also tested since it encodes the kinase upstream of *FAB1* in the phosphoinositide pathway and also caused

Relevant genotype

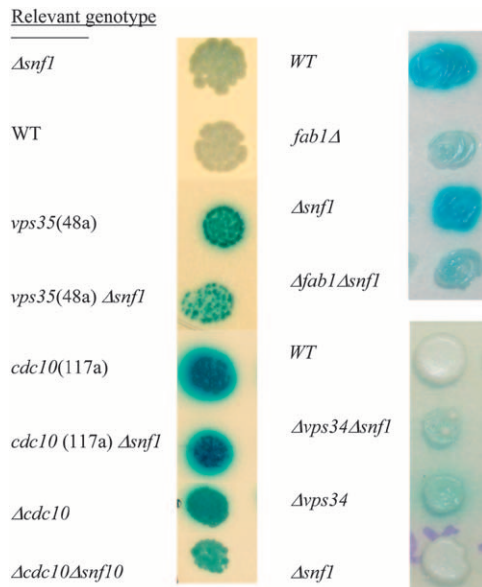|	
Δsnf1	*WT*
WT	*fab1Δ*
vps35(48a)	*Δsnf1*
vps35(48a) *Δsnf1*	*Δfab1Δsnf1*
cdc10(117a)	*WT*
cdc10 (117a) *Δsnf1*	*Δvps34Δsnf1*
Δcdc10	*Δvps34*
Δcdc10Δsnf10	*Δsnf1*

FIGURE 7.—*SNF1*-independence of constitutive *ADH2/lacZ* expression. Cells were serially diluted and spotted or patched onto X-gal-8% glucose plates and incubated at 30° or the permissive temperature for ts strains. The strains used are described in the text and MATERIALS AND METHODS. Only one dilution is shown.

constitutive *ADH2* expression. For *fab1* mutant strains we were unable to recover *snf1* deletion transformants so JSY24 *Δsnf1::kanmx* was mated to VBY20 *Δfab1::nat1* or VBY20 *Δvps34::nat1*, the heterozygous diploids were sporulated, and haploid double mutants were identified by their resistance to both nourseothricin and G418. In this manner *vps34snf1* double mutants were easily recovered. However, the heterozygous *FAB1/fab1::nat1 snf1::kanmx/SNF1* diploid gave very poor sporulation and no *fab1snf1* mutants, possibly due to a germination defect. To overcome this problem, the *fab1::nat1/FAB1 snf1::kanmx/SNF1* diploid was transformed with a *FAB1-URA3*-CEN plasmid. The resulting diploid gave better sporulation and many *fab1snf1* double mutants, all of which were Ura⁺, indicating that they contained the *FAB1-URA3* plasmid. Selection on 5′-FOA yielded *fab1snf1* double mutants lacking the *FAB1* gene. The *fab1snf1* and *vps34snf1* double mutants were temperature sensitive for growth (indicating *fab1* or *vps34* mutations) and glycerol negative (*snf1*), yet exhibited constitutive *ADH2/lacZ* expression. The constitutive *ADH2* expression of *vps35* and *cdc10* mutants was also *SNF1*-independent (Figure 7). Thus, all these mutations represent alterations in pathway(s) distinct from repression mediated by Glc7.Reg1.Bmh1 and by Mot1 (see below). Mutations in any of these genes cause constitutive *ADH2* expression only in the presence of an active *SNF1* allele (DOMBEK *et al.* 1993, 2004).

ADR7 (mutant 13a)—MOT1: ts mutant 13**a** did not fall into 1 of the 12 complementation groups. However, it failed to complement strains containing mutations in *ADR7* that were isolated in a previous screen for constitutive *ADH2* expression in the presence of an overexpressed, normally inactive *ADR1-220* allele (KARNITZ *et al.* 1992). Both *ADR7* mutant alleles, like mutant 13**a**, are temperature sensitive.

ADR7 (mutant 13**a**) was cloned by complementing the ts and At^R (antimycin A) mutant phenotypes of strain S54 as described in MATERIALS AND METHODS. Sequence analysis of the complementing fragment revealed that it contains *MOT1*. The cloned *YCp50-MOT1* library plasmid also complements the VMY13**a** mutant phenotypes, consistent with genetic analysis showing allelism between *ADR7* and mutant13**a** (V. VORONKOVA, unpublished data).

Allelism between *MOT1* and *ADR7* was demonstrated by integrative transformation and subsequent genetic analysis as described in MATERIAL AND METHODS (V. VORONKOVA, unpublished data). These results indicated that *adr7-1* is closely linked to or within *MOT1*. Since the transcriptional defects of *adr7-1* and mutant 13**a** are similar to those of other *mot1* temperature-sensitive alleles, the mutations are most likely in *MOT1* itself.

Mot1 is a general repressor of Pol II transcription and acts by displacing TBP from the TATA box in an ATP-dependent fashion (AUBLE and HAHN 1993; AUBLE *et al.* 1994, 1997). The fact that in two different screens for constitutive *ADH2* expression we isolated *MOT1* mutants confirms that Mot1 is important for maintaining glucose repression of *ADH2* expression.

mot1 (*adr7*)-dependent constitutive *ADH2* activity requires *SNF1* and acts independently of the *ADH2-10* deletion: Since mutations in *MOT1* are known to cause gene expression that is partially independent of normal activator requirements *in vivo* (DAVIS *et al.* 1992), it is important to know if the constitutive *ADH2* expression acts through the normal pathway of derepression, which requires Adr1 and Snf1. Since the effects of *adr7-1* are stronger in the presence of *ADR1-220*, which is an overexpressed, truncated allele of *ADR1* that has very low activity in a wild-type strain (KARNITZ *et al.* 1992), we used a strain deleted for the wild-type copy of *ADR1* and carrying a plasmid expressing either *ADR1-220* or no *ADR1*. As shown in Table 4 the ADHII activity was low in the parent strain, SSH35, with *ADR1-220*. Activity increased about sixfold in the *adr7-1* mutant in an *ADR1-220*-dependent fashion (compare lines 1–3).

The activity decreased to the wild-type level in the absence of *SNF1* (line 4). These data indicate that the constitutive *ADH2* expression caused by a mutation in *MOT1* requires both *ADR1* and *SNF1*. Even the weak *ADR1*-independent constitutive expression observed in the *adr7-1* mutant is *SNF1* dependent (compare lines 3 and 5). Although *MOT1* (*ADR7*) behaves genetically like a repressor of *ADH2* expression, it does not have sequence-specific DNA-binding properties and is thus unlikely to bind the *ADH2* promoter. Nonetheless, its activity requires DNA upstream of the TATA box (DARST

TABLE 4

ADHII activities in *adr7-1 (mot1)* mutant strains

| Line | Strain | Genotype | | | | ADHII activity (R)[b] | At^R c |
		MOT1	SNF1	ADR1[a]	ADH2		
1	SSH35	WT	WT	*220*	WT	29	−
2	S54	*adr7-1*	WT	*220*	WT	195	+++
3	S54	*adr7-1*	WT	Δ	WT	50	−+
4	S54ΔS	*adr7-1*	Δ	*220*	WT	20	−
5	S54ΔS	*adr7-1*	Δ	Δ	WT	22	−
6	LK13	WT	WT	*220*	*ADH2-10*	*240*	+++
7	LV14	*adr7-1*	WT	*220*	*ADH2-10*	*500*	+++
8	LK13ΔS	WT	Δ	*220*	*ADH2-10*	*18*	−
9	LV14ΔS	*adr7-1*	Δ	*220*	*ADH2-10*	*75*	−+
10	LK13ΔS	WT	Δ	Δ	*ADH2-10*	*15*	−
11	LV14ΔS	*adr7-1*	Δ	Δ	*ADH2-10*	*50*	−+

Cells were grown in synthetic medium containing 5% glucose and crude cell extracts were assayed for ADH activity as described in MATERIALS AND METHODS.

[a] The *ADR1-220* allele was present on a *CEN4-TRP1* plasmid (KARNITZ *et al.* 1992). In strains denoted Δ in the *ADR1* column the pRS314 vector was present.

[b] The ADH activity is the average of triplicate measurements that had a standard deviation of ~10%.

[c] Growth in the presence of the respiratory inhibitor Antimycin A (At^R) is indicative of constitutive *ADH2* expression (KARNITZ *et al.* 1992). Growth was scored as good growth (+++), very poor growth (−+), or no growth (−) after replica plating patches from a plate containing synthetic Trp⁻ medium supplemented with 5% glucose.

et al. 2001). *ADH2-10* is a 12-bp deletion/insertion mutation in the *ADH2* promoter, located 40 bp upstream of the TATA box (−104 to −110). *ADH2-10* is constitutively active in the presence of the *ADR1-220* allele, whereas wild-type *ADH2* is not (KARNITZ *et al.* 1992). This phenotype is identical to the phenotype caused by loss of Mot1 activity (Table 4, compare lines 2 and 6). If Mot1 activity at the *ADH2* promoter requires the sequence deleted in *ADH2-10*, then a strain containing both the *adr7-1* (*mot1*) allele and the *ADH2-10* promoter mutation should have no more activity than either single mutant. If Mot1 acts through a different location in the *ADH2* promoter (directly at the TATA box, for example) or elsewhere in the repression pathway, then constitutive activity should be additive or greater in the *ADH2-10* strain with a mutation in *adr7-1*. To test these alternatives, an *adr7-1 ADH2-10* double mutant was constructed and *ADH2* expression was assayed. ADHII activity in the double mutant is higher than in either single mutant alone (Table 4, compare lines 7, 6, and 2). The constitutive *ADH2* expression in the *ADH2-10* mutant and in the *adr7-1ADH2-10* double mutant is *SNF1* dependent, although the double mutant did exhibit a significantly higher level of *SNF1*-independent *ADH2* expression than either single mutant (Table 4, compare lines 6 and 7 to lines 8 and 9). Deleting *ADR1* in addition to *SNF1* in the *mot1* or *mot1ADH2-10* mutants caused little further decrease in *ADH2* expression, indicating that the *SNF1*-independent activity is also *ADR1* independent (compare lines 10 and 11 to lines 8 and 9). Thus, *ADH2-10* and *MOT1* appear to affect *ADH2* repression through different mechanisms, and the constitutive activity in both mutants requires Snf1.

Adr1 levels in constitutive mutants: Since several of the VMY mutants are in vacuolar trafficking pathways and the vacuole is associated with protein degradation, we considered the possibility that the constitutive activity of *ADH2* in the newly isolated mutant strains could be due to an increased steady state level of the Adr1 activator under repressed conditions. Very high levels of Adr1 can cause constitutive *ADH2* expression (DENIS 1987; IRANI *et al.* 1987), and in the multicopy *ADR1* strains used, Adr1 levels are elevated to derepressed levels. Although degradation of nonsecretory pathway proteins by the vacuole is unusual, it is not without precedent. A gluconeogenic enzyme that is required only under low-glucose conditions is degraded by import into vesicles for transport to the vacuole when glucose is abundant (BROWN *et al.* 2002). To test this possibility, protein extracts of wild-type (JSY24) and VMY mutant strains were prepared and Western blots were performed. The amount of Adr1 under repressed conditions was determined by quantitative Western blotting for two or three mutants in each of the 12 complementation groups. Although some mutant strains showed two- to threefold elevated Adr1 levels, there were no consistent differences between mutant and wild-type strains (Figure 8 and supplemental Table 2 at http://www.genetics.org/supplemental/). We conclude that enhanced expression or stability of Adr1 is not responsible for the constitutive *ADH2* expression in the mutants.

DISCUSSION

We identified and characterized a large collection of mutants that exhibit constitutive *ADH2/lacZ* expression
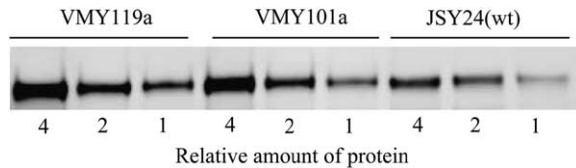
FIGURE 8.—Adr1 levels in *ADH2* constitutive mutants. Extracts from mutant and wild-type (wt) strains were analyzed by Western blot as described in MATERIALS AND METHODS. Two representatives from each complementation group were analyzed. Shown here are *ADR14*(VMY119**a**) and *ADR16* (VMY101**a**). Equal amounts of protein, in decreasing amounts of four-, two- and onefold, were loaded for each strain.

in the presence of additional copies of the regulatory gene *ADR1*. In most of the mutants the endogenous *ADH2* locus was also expressed constitutively and constitutive *ADH2* expression was dependent on the major activator Adr1. Mutations in three complementation groups also partially alleviate *SUC2* repression suggesting that these three genes have a general role in glucose repression.

Mutations in many genes not belonging to one of the 12 complementation groups also led to low-level resistance to glucose repression. One of these, mutant 13**a**, is allelic to *ADR7*, and another, 78**a**, is allelic to *ADR8*, both of which were identified in a previous genetic selection for glucose-insensitive *ADH2* expression in the presence of an overexpressed, inactive *ADR1-220* allele (KARNITZ *et al.* 1992). Since only single representatives of these genes were identified, and many other genes are apparently represented only once in our collection, the screen is far from saturated. Thus, alteration of cellular physiology in numerous ways can lead to constitutive *ADH2* expression when the requirement for the major activator Adr1 is satisfied. This may explain why *ADR1* is normally expressed at very low levels under repressed growth conditions.

Cloning *ADR7* (mutant 13**a**) revealed that it encodes the general repressor of polymerase II transcription, Mot1. *MOT1* was identified in genetic screens for activation of transcription in the absence of an activator or a UAS sequence (DAVIS *et al.* 1992) and as an inhibitor of transcription *in vitro* (AUBLE and HAHN 1993). Mot1 can also act as an activator of basal transcription *in vitro*, and *in vivo* some genes are activated by the absence of *MOT1* (COLLART 1996). *In vitro* Mot1 acts to displace TBP from a TATA box in an ATP-dependent fashion (AUBLE *et al.* 1994). Mot1 interacts with the surface of TBP that interacts with DNA and thus acts as a competitive inhibitor of binding to a TATA box (DARST *et al.* 2001). *In vivo* Mot1 is thought to displace TBP from weak or nonproductive sites and allow TBP to function more effectively from productive promoter sites (MULDROW *et al.* 1999).

The finding that *ADR7* and *MOT1* are allelic suggests that TBP binding to the *ADH2* promoter may be inhibited by Mot1 during glucose repression. This idea is consistent with recent evidence indicating that Mot1 association with transcription components is regulated by environmental stress (GEISBERG and STRUHL 2004). After glucose depletion, the level or activity of Mot1 could be altered, resulting in expression of *ADR1*-dependent genes. Alternatively, the role of Mot1 in *ADH2* repression may act through chromatin structure of the *ADH2* promoter (VERDONE *et al.* 1996) since Mot1 has a role in chromatin remodeling independent of its interaction with TBP (TOPALIDOU *et al.* 2004). We failed to detect Mot1 at the *ADH2* promoter using chromatin immunoprecipitation (C. TACHIBANA, unpublished data), suggesting that Mot1 may not act directly to repress TBP binding at the *ADH2* promoter. For this reason we favor the possibility that Mot1 allows Snf1-dependent Adr1 binding and activation through an effect on chromatin structure, which is known to influence Adr1 binding (VERDONE *et al.* 1996).

We cloned genes representing three other complementation groups not previously implicated in glucose repression. One of these, *ADR22*, is the *FAB1* gene. *FAB1* encodes PtdIns (3′)-P-(5′)-kinase (YAMAMOTO *et al.* 1995; BONANGELINO *et al.* 2002; GARY *et al.* 2002). Fab1 is involved in cargo-selective sorting of membrane proteins to the lumen of the vacuole (ODORIZZI *et al.* 2000), a process that requires its kinase activity. *fab1* mutants have other defects as well, including a defect in nuclear morphology and osmotolerance (BONANGELINO, *et al.* 2002) suggesting that it may have a role in cellular signaling. A second connection between *ADH2* regulation and the vacuolar sorting pathway is the identification of *VPS35* (*ADR11*) in our screen. *VPS35* encodes a protein that is part of the retromer complex involved in sorting hydrolases to the vacuole (PARAVICINI *et al.* 1992; SEAMAN *et al.* 1998). The identification of mutations that alleviate glucose repression of *ADH2* expression in a second gene in the sorting pathway suggests that vacuolar metabolism, rather than an unidentified function of Fab1, may cause relief from repression. However, impaired degradation of the major *ADH2* activator, Adr1, is not responsible for constitutive *ADH2* activation since the levels of Adr1 in wild-type *vs.* mutant whole-cell extracts are nearly equivalent.

An alternative possibility is that the effect of Fab1 on *ADH2* expression is caused by an unknown cell-signaling function of Fab1. The substrate of the Fab1 kinase reaction, PtdIns (3′)-P, is the product of a PtdIns-3′-kinase encoded by *VPS34* and deletion of *VPS34* also causes constitutive *ADH2* expression. This result suggests that the kinase activity of Fab1 is important for maintaining glucose repression, an interpretation consistent with the phenotype of a kinase-dead allele. Together, the results suggest that depleting the pool of phosphatidylinositol-3′,5′-phosphate relieves glucose repression. The pool of di- and triphosphoinositides in yeast is dramatically decreased when the energy charge of the cell is lowered, such as by glucose starvation
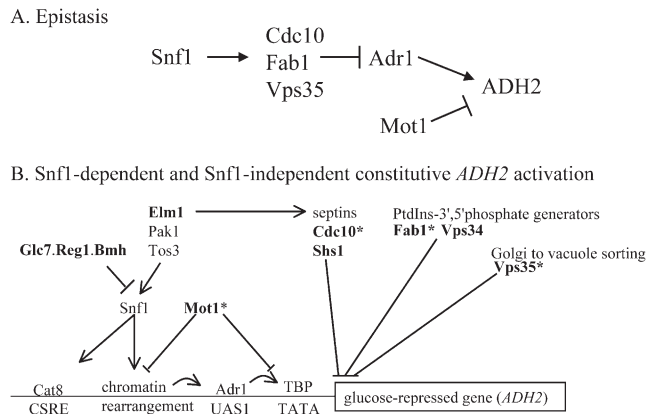
A. Epistasis

B. Snf1-dependent and Snf1-independent constitutive *ADH2* activation

FIGURE 9.—Interactions between factors affecting *ADH2* expression. (A) Epistasis relationships between *ADR1*, *SNF1*, and *CDC10*, *FAB1*, *VPS35*, and *MOT1* in *ADH2* expression. Positive and negative interactions are shown by arrows and bars, respectively. (B) A model of *ADH2* expression incorporating new regulatory genes. Boldface type indicates mutants are *ADH2*-constitutive. The asterisks indicate mutants isolated in this screen or in accompanying studies.

(LESTER and STEINER 1968). To date, however, these signaling molecules have not been associated in a causal manner with glucose repression.

Polyphosphoinositides have been implicated in gene expression through their effects on chromatin-remodeling activities (ODOM *et al.* 2000; SAIARDI *et al.* 2000; STEGER *et al.* 2003). Since Adr1-dependent but transcription-independent chromatin remodeling accompanies *ADH2* derepression (VERDONE *et al.* 1997), if aberrant chromatin structure were induced in the *fab1* mutant it could contribute to constitutive *ADH2* expression.

The isolation of *CDC10* and subsequent demonstration that two other genes involved in septin formation, *SHS1* and *ELM1*, affect *ADH2* regulation is the first suggestion that genes affecting 10-nm filaments may be involved in glucose signaling. Analysis of other septin mutants may reveal whether this is a direct and specific effect of these particular septin components or is an indirect consequence of the loss of septin assembly. Elm1 kinase is also an upstream kinase for Snf1, raising the possibility that the constitutive *ADH2* expression in the *ELM1* mutants acts through the *SNF1* pathway of *ADH2* regulation. Both loss-of-function and gain-of-function alleles of *ELM1* cause constitutive *ADH2* expression, suggesting that the activity of this pathway is very sensitive to the level of Elm1 activity since either too much or too little Elm1 causes a mutant phenotype. Mutations in two other Snf1 upstream kinases, Pak1 and Tos3, did not cause a constitutive *ADH2* phenotype (N. KACHEROVSKY, unpublished data). Thus, it appears likely that the constitutive activity of *ELM1* mutants acts through the septin pathway rather than through a *SNF1*-dependent activation pathway.

Our data provide the first evidence suggesting the involvement of the PtdIns (3′)-P-(5′)-kinase, Fab1, and septin assembly in glucose repression of the key gene involved in ethanol metabolism, *ADH2*. The possible genetic interactions between these genes, Mot1, Adr1, and Snf1 are shown in Figure 9A. An important question remaining is whether these genes act in a direct or an indirect manner to mediate repression of gene expression.

Together, these newly identified mutants outline at least two avenues from glucose depletion to transcriptional response that are dependent on Adr1 (Figure 9B). One involves the AMPkinase Snf1 and the repressor and chromatin remodeling enzyme Mot1. In the other, Snf1-independent route, the contributions of Cdc10, Fab1, and Vps35 to transcriptional regulation are less well-characterized, but may involve cell sensing and signaling of glucose and other stress conditions.

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